Loss of PKBβ/Akt2 predisposes mice to ovarian cyst formation and increases the severity of polycystic ovary formation in vivo

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Abstract

Ovarian cysts affect women of all ages and decrease fertility. In particular, polycystic ovarian syndrome (PCOS), with multiple follicular cysts, affects 5-10% of women of reproductive age and can result in infertility. Current non-invasive treatments for PCOS can resolve cysts and restore fertility, but unresponsive patients must undergo severe ovarian wedge resection and resort to in vitro fertilization. PCOS is related to the deregulation of leutinizing hormone (LH) signaling at various levels of the hypothalamic-pituitary-ovarian axis and resultant hyper-production of androgens. As insulin resistance and compensatory hyperinsulemia are observed in 50-70% of PCOS patients, deregulated insulin signaling in the ovary is considered an important factor in the disease. Here we report that aged mice, specifically lacking the PKBβ/AKT2 isoform that is crucial for insulin signaling, develop increased testosterone levels and ovarian cysts also observed in insulin resistant PCOS patients. Young PKBβ/AKT2 knockout mice subjected to a mouse model of PCOS, by treatment with LH, exhibited a cyst area threefold greater than controls, but without hyperinsulemia. Thus, loss of PKBβ/AKT2 may predispose mice to ovarian cysts independent of hyperactive insulin signaling. Targeted therapeutic augmentation of specific PKBβ/AKT2 signaling may therefore provide a new avenue for the treatment and management of ovarian cysts.
Introduction

Ovarian cysts are the most common female reproductive abnormality in women of all ages [for review see (Goodarzi et al., 2011)]. Simple cysts have been poorly studied as they usually result in only minor discomfort, are generally slow growing and benign, and can resolve without treatment. Large simple cysts commonly detected in older women, where maintaining fertility is often not an issue, can be dealt with by uni- or bi-lateral oophorectomy surgery.

In contrast, polycystic ovarian syndrome (PCOS), characterized by multiple small cysts in the ovary, is observed from puberty, affects 5-10% of women of reproductive age and can result in infertility. PCOS can be resolved in some cases by lifestyle changes, weight loss and by treatments ranging from insulin-sensitizing drugs to hormone supplementation. In particularly refractory cases and when continued fertility is an issue, ovarian wedge resection may be necessary and in vitro fertilization performed.

PCOS has been associated with deregulation of LH signaling, which can occur at various levels of the hypothalamic-pituitary-ovarian (HPO) axis and stimulate hyperproduction of androgens (Goodarzi et al., 2011). Deregulation may occur through hyperstimulation of the pituitary by the hypothalamus, leading to increased LH release or commonly by increased sensitivity of ovarian LH-sensitive thecal cells to LH. In both cases, abnormal leutinizing hormone receptor (LHR) signaling within the LH-responsive ovarian thecal cells results in an increased steroidogenic response
and androgen production (Strauss, 2003). Genetic manipulation of LHR signaling in mice or modeling PCOS by administration of exogenous LH or androgens, results in ovarian cyst development, as well as the hallmark of increased testosterone production (Familiari G, 1985; Bogovich, 1987; Huhtaniemi et al., 2006; Manneras et al., 2007). This is also observed in PCOS patients who display abnormalities in thecal steroidogenesis (Nelson et al., 1999).

Deregulation of the canonical thecal steroidogenic pathway, from LH receptor activation through protein kinase A (PKA) and the cAMP response element-binding protein (CREB) transcription factor appears central to cyst development (Johnson and Sen, 1989; Tremblay et al., 2002; Towns et al., 2005; Towns and Menon, 2005). However, a contribution of non-classical cAMP-independent signaling, like protein kinase C (PKC), mitogen-activated protein kinases/extracellular-signal-regulated kinases (ERKs) and phosphoinositol-3-kinase - protein kinase B/Akt (PI3K-PKB/Akt), appears probable in PCOS, as defects in these signaling pathways are known to affect LH-mediated androgenesis (Manna et al., 2006; Diamanti-Kandarakis et al., 2008). In particular, several further observations point to an involvement of insulin receptor (InsR) signaling in PCOS. Defects in InsR phosphorylation (Dunaif et al., 1995) and further genetic lesions in this pathway, affecting InsR, PKBβ/Akt2 and downstream glycogen synthase kinase 3 beta (GSK3β) are found in PCOS patients (George et al., 2004; Tan et al., 2007; Goodarzi et al., 2008; Mukherjee et al., 2009). Insulin can act as a ‘co-gonadotrophin’ and exogenous administration of LH in rodent models of PCOS can exacerbate PCOS cyst formation (Poretsky et al., 1992). As many as 50-70% of PCOS patients display insulin resistance (IR) and compensatory hyperinsulemia (Diamanti-Kandarakis et al., 2008). Furthermore, up to 60% of PCOS patients are obese, which is the most common factor leading to IR and can result in
decreased InsR expression and post-receptor dysfunction in downstream kinase activation (Mlinar et al., 2007; Vrbikova and Hainer, 2009).

There are three isoforms of PKB, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3, which display both redundant and specific functions, of which PKBβ/Akt2 has specific and crucial functions in metabolism and insulin signaling (Dummler and Hemmings, 2007). Indeed, PKBβ/Akt2 knockout (KO) mice progressively develop a diabetes-like phenotype, displaying peripheral IR and compensatory hyperinsulemia (Cho et al., 2001a; Garofalo et al., 2003; Dummler et al., 2006). We report that aged female PKBβ/Akt2 KO mice develop severe ovarian cysts. Consistent with an involvement of LH signaling in the formation of these cysts, we observed hyperplasia of the thecal-interstitial in these KO mice, which increased with age and correlated with the severity and size of the cysts. Because targeted therapeutic augmentation of specific PKBβ/Akt2 activities could provide a new avenue for the treatment and management of ovarian cysts, we set out to plot more precisely the involvement of PKBβ/Akt2 in cyst development, by analysis of cysts from aged PKBβ/Akt2 KO mice and subjecting young PKBβ/Akt2 KO mice to a PCOS model.

Abbreviations: CREB; cAMP response element-binding protein, ERK; mitogen-activated protein kinase/extracellular-signal-regulated kinase 1/2, GnRHant; gonadotrophin-releasing hormone antagonist, GSK3α/β; glycogen synthase kinase 3alpha/beta; H&E; Haematoxylin and Eosin, HPO; hypothalamic-pituitary-ovarian, IHC; immunohistochemistry, InsR; insulin receptor, IR; Insulin resistance, KO; knockout, LH; leutinizing hormone, LHR; leutinizing hormone receptor, PCOS; polycystic ovarian syndrome, PI3K; phosphoinositol-3-kinase, PKA; protein kinase A, PKBα/Akt1; protein kinase B alpha/ v-Akt murine thyoma viral oncogene 1,
PKBβ/Akt2; protein kinase B beta/v-Akt murine thyoma viral oncogene 2,  
PKBγ/Akt3; protein kinase B gamma/ v-Akt murine thyoma viral oncogene 3, PKC;  
protein kinase C (PKC), StAR; steroid acute regulatory protein, WT; wild-type, 3β- 
HSD; 3β-hydroxysteroid dehydrogenase.

**Results**

**Specific ablation of the PKBβ/Akt2 isoform leads to the development of severe ovarian cysts in aged mice.**

Examination of aged female mice with distended abdomens led to the discovery that mice lacking PKBβ/Akt2 but not wild-type (WT) mice develop severe ovarian cysts (Figure1A, B). Cysts developed in almost 80% of PKBβ/Akt2 KO mice, were generally filled with serous fluid with bi-lateral presentation observed in 50% of cases (Table 1). Cysts were absent in WT mice or were present as small follicular cysts with a uni-lateral distribution (Figure 1B). Larger cysts were also observed in older PKBβ/Akt2 KO mice, suggesting an increase in size with age. Ovaries of PKBα/Akt1 KO mice, the other major PKB isoform found in the ovary, were similar to WT mice, i.e. were small with a uni-lateral presentation of follicular cysts (Figure1B and Table 2). This suggests that severe cyst development was due to specific loss of the PKBβ/Akt2 isoform.
Figure 1: Specific loss of PKBβ/Akt2 in aged mice results in the development of severe ovarian cysts with an increase in the thecal-interstitial cell population. (A) 91-120 week-old WT (i) and PKBβ/Akt2 KO (ii) mice with distended abdomens. Cystic ovaries isolated from these WT (i) and PKBα/Akt1 KO (iii) mice show no atresia or small ovarian cyst formation, whilst PKBβ/Akt2 KO mice show severe ovarian cyst formation. (C) Increased stromal accumulation in early 91-week-old (i, iii) and late 120-week-old (ii, iv) aged PKBβ/Akt2 KO mice, shown by hematoxylin and eosin staining (i, ii), and as a consequence of increased thecal-interstitial hyperplasia, as indicated by positive vimentin immunohistochemical staining (iii, iv). x40 magnification.
Ovarian cysts in aged PKBβ/Akt2 KO mice are characterized by thecal-interstitial hyperplasia.

To identify histological abnormalities that may precede cyst development, cysts isolated from PKBβ/Akt2 KO mice were examined by haematoxylin and eosin (H&E) and immunohistochemical staining. H&E staining showed a severe reduction or lack of corpus luteum in aged ovaries, which also stained negative for the granulosa cell marker anti-mullerian inhibiting substance (Supplemental Figure 1A, B). This suggests a cessation of estrous cycling. Hyperplasia of spindle-like stromal cells that increased with ovarian cyst size was observed in PKBβ/Akt2 KO mice. Positive staining for vimentin identified these cells as part of the thecal-interstitial cell population (Figure 1C).

Table 1: Overview of ovarian cyst incidence and characteristics from aged 90-120 week-old female WT, PKBβ/Akt2 KO and PKBα/Akt1 KO mice.

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>PKB α/Akt1</th>
<th>PKB β/Akt2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Of Animals</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>No. Of Animals With Cysts</td>
<td>2 (33%)</td>
<td>7 (50%)</td>
</tr>
<tr>
<td>-Unilateral/Bilateral</td>
<td>2/0</td>
<td>5/2</td>
</tr>
<tr>
<td>-Maximum Diameter (mm)</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>-Average Diameter (mm)</td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>-Earliest Detection (wks)</td>
<td>98</td>
<td>91</td>
</tr>
<tr>
<td>Mean Age Of Animals (wks)</td>
<td>~112</td>
<td>~108</td>
</tr>
<tr>
<td>Age Range of Animals (wks)</td>
<td>98-121</td>
<td>91-128</td>
</tr>
</tbody>
</table>

Disease Models & Mechanisms
Figure 2: Aged WT and PKBβ/Akt2 KO mice show active leutinizing hormone (LH) signaling with ovarian cysts from PKBβ/Akt2 KO mice displaying increased steroidogenic signaling, lipid accumulation and serum testosterone. (A) WT and PKBβ/Akt2 KO mice exhibit circulating serum leutinizing hormone (LH) with no significant difference in hormone levels. (B) PKBβ/Akt2 KO ovarian cysts display both active CREB (i-iii) and ERK (iv-vi) signaling required for steroidogenesis. ERK is located at the cystic lumen (arrows) and increases with the severity of the cysts and age of the KO mice (ii, iii & v, vi) but is absent in WT mice (i, iv). Magnification x100. (C) PKBβ/Akt2 ovarian cysts (ii, iv) display increased lipid accumulation adjacent to the cystic lumen, a prerequisite for conversion to steroids; this is absent in WT mice (i, iii). Magnification x40 and x100. (D) Consistent with increased active steroidogenesis, PKBβ/Akt2 KO mice show increased serum testosterone levels compared with WT mice.
Ovarian cysts in aged PKBβ/Akt2 KO mice show increased steroidogenic capacity.

The potential for active steroidogenic signaling by thecal-interstitial cells in cystic ovaries was indicated by hyperplasia in cystic ovaries of the LH-responsive thecal-interstitial cells responsible for ovarian steroidogenesis and absence of granulosa cells, important for controlling LH levels via negative feedback signaling on the pituitary (Massicotte et al., 1984; Couzinnet B, 1993). Circulating LH was found in serum with no significant difference in hormone levels between WT and KO animals (Figure 2A). LH activation of LHR is crucial for steroidogenesis, by triggering the phosphorylation of CREB transcription factor and ERK kinase (Salvador et al., 2002); this mediates transcription of enzymes and intracellular signaling that regulate uptake of cholesterol and its enzymatic conversion to C-19 androgens. Cystic ovaries from PKBβ/Akt2 KO mice displayed activating phosphorylation of CREB and ERK, with strong ERK activation commonly observed in cells adjacent to the cystic lumen (Figure 2B). In contrast, WT ovaries, whilst displaying CREB activation, were devoid of ERK. Consistent with the activation of ERK in PKBβ/Akt2 KO cysts, lipids were observed in cells surrounding the cystic lumen of PKBβ/Akt2 KO mice but were absent in WT mice, indicating functional uptake of cholesterol for steroidogenesis in PKBβ/Akt2 KO mice only (Figure 2B). To determine whether the hyperplastic thecal-interstitial cell population observed in ovarian cysts from PKBβ/Akt2 KO mice is steroidogenically active and producing bioactive androgens, serum testosterone levels were measured in WT and PKBβ/Akt2 KO mice. Whereas WT showed generally low testosterone levels, aged PKBβ/Akt2 KO mice consistently showed a twofold higher content (Figure 2C).
Figure 3: Loss of PKBβ/Akt2 has no significant impact on normal ovarian steroidogenic signaling or reproductive function in young WT or PKBβ/Akt2 KO mice. (A) Steroidogenic signaling through CREB (i, ii) and ERK (iii, iv) in WT (i, iii) and PKBβ/Akt2 KO (iii, iv) mice. (B) Circulating serum hormone levels of testosterone (i) and estrodiol (ii) in WT and PKBβ/Akt2 KO animals. (C) PKBβ/Akt2 KO mice are fertile and produce litter sizes similar to matings from WT animals.
Young PKBβ/Akt2-ablated mice display normal steroidogenic and reproductive functions.

As steroidogenesis was found to be abnormal in cysts from aged PKBβ/Akt2 KO mice, steroidogenesis and reproduction in young (4- to 8-week-old) PKBβ/Akt2 KO mice were also examined. Neither function was compromised by loss of PKBβ/Akt2 (Figure 3). There was no difference in the activation of steroidogenic signaling as shown by phosphorylation of CREB and ERK between WT and PKBβ/Akt2 KO ovaries from young animals (Figure 3A). The thecal-interstitial population showed low to moderate activation of both proteins compared to aged cystic ovaries and testosterone and estrogen serum levels were equivalent in young WT and PKBβ/Akt2 KO mice (Figure 3B). Finally, reproduction as assessed by litter sizes was not significantly different between young WT and PKBβ/Akt2 KO animals (Figure 3C). Thus compensatory mechanisms in the HPO axis appear to be sufficient to maintain normal ovarian function in young PKBβ/Akt2 KO mice.

Induction of PCOS via tonic LH stimulation in young PKBβ/Akt2 KO mice increases the severity of polycystic ovaries.

Given that aged KO mice showed severe cystic development and enhanced activation of androgenic steroidogenesis in thecal-interstitium compared with WT animals, but young KO mice showed no such dysfunctions, it was possible to design an experiment using LH treatment of young mice to plot more precisely the importance of PKBβ/Akt2 in the chain of events leading to ovarian steroidogenesis and cyst development. Tonic LH treatment hyperstimulates LH signaling, mimics the PCOS setting, produces features of PCOS pathology, including increased steroidogenic signaling and testosterone production, and results in cyst formation (Poretsky et al.,
1992). To counter a possible effect on LH level of an increase in negative feedback to the pituitary (Poretsky et al., 1992), LH stimulation was also administered in the presence of a gonadotrophin-releasing hormone antagonist (GnRHAnt).

Consistent with previous reports (Poretsky et al., 1992), administration of LH with or without GnRHAnt led to the development of haemorrhagic follicular cysts in young WT mice, whilst treatment with either the dosing vehicle or GnRHAnt alone did not (Figure 4A). In marked contrast, treatment of young PKBβ/Akt2 KO mice with LH alone or with GnRHAnt led to an approximate threefold increase in cystic area in the ovaries compared with treated WT; cyst development was not affected by the vehicle or GnRHAnt treatment alone (Figure 4A). CREB was activated and supported steroidogenesis in the ovaries of both young WT and PKBβ/Akt2 KO mice treated with LH (Figure 4B, i-iv), and activated ERK was strongly expressed in the thecal cells adjacent to the cystic lumen (Figure 4B, v-viii), reminiscent of the pattern in the cysts of aged PKBβ/Akt2 KO mice. In the young mice, ERK activation was particularly strong around cysts in PKBβ/Akt2 KO ovaries and, unlike in WT ovaries, ERK activation was also strong in thecal cells surrounding large follicles (Figure 4B, vi, viii, arrows). Such CREB and ERK activity could support increased androgen steroidogenesis, follicular degeneration and cyst development, thus causing the increase in ovarian cyst area in young PKBβ/Akt2 null ovaries. Consistent with this, 3β-HSD staining revealed active steroidogenesis in the thecal-interstitial population of all cystic ovaries (Figure 4C, v-viii). Lipid accumulation was higher in this steroidogenic population of PKBβ/Akt2 KO ovaries than in WT (Figure 4C, i-iv), indicating increased cholesterol uptake in young PKBβ/Akt2 KO mice that could support androgen production in an environment of ERK activation.
Figure 4: Induction of PCOS via tonic LH administration results in an increase severity of ovarian cysts observed in PKBβ/Akt2 KO ovaries, with formation of cysts associated with ERK activation and lipid accumulation in steroidogenically active ovaries. (A) PKBβ/Akt2 KO ovaries showed an approximately three-fold increase in ovarian cyst area in LH treated ovaries (vi, viii) compared to WT (v, vii), independent of administration of a gonadotrophin releasing hormone antagonist. Treatment of WT and PKBβ/Akt2 KO mice with vehicle (i, ii) or gonadotrophin releasing hormone antagonist (iii, iv) alone had no effect on cyst formation. (B) Steroidogenic signaling was active and seen in both ovaries from WT (i, iii, v, vii) and PKBβ/Akt2 KO (ii, iv, vi, viii) treated with LH. ERK however was also observed strongly active with increased theca thickness surrounding large follicles predominantly in PKBβ/Akt2 KO ovaries (arrows). (C) Increased lipid accumulation in ovaries treated with LH was also observed in PKBβ/Akt2 KO mice (ii, iv) in areas with active androgen steroidogenesis [indicated by active 3β-HSD staining (v-viii)] compared to WT (i, iii). All magnifications for IHC are 100x.

As described above, insulin can act as a ‘co-gonadotrophin’ that can increase the severity of cysts, and PKBβ/Akt2 KO mice develop a progressive diabetes-like syndrome with hyperinsulemia. Importantly, despite the marked difference in PCOS-like symptoms young PKBβ/Akt2 KO mice did not display hyperinsulemia under the
random-fed conditions of the experiment, with no significant difference observed in circulating insulin levels between young WT and PKBβ/Akt2 KO animals (Suppl.Fig 2). This lack of hyperinsulemia is probably due to the young age and genetic background, as female PKBβ/Akt2 KO mice on a genetic background displaying a more severe insulin resistant phenotype, have a weaker insulin resistance phenotype than male mice and only mild differences in circulating insulin levels up to 24 weeks of age (Garofalo et al., 2003). Thus, the high prevalence of cysts in young mice following PKBβ/Akt2 ablation does not appear the result of hyperstimulation of ovarian InsR.

**Figure 5: Model Outlining The Pro-Androgenic Contributions Of PKBβ/Akt2 Loss To LH-Driven Pathogenic Cyst Formation.** In the normal physiology, the negative feedback along the hypothalamic-pituitary-ovarian axis ensures steroidogenic signaling is tightly controlled. In pathogenic scenarios of ovarian cyst formation like formation of simple cysts in aged mice or cyst formation in PCOS, a lack of PKBβ/Akt2 combined with increased LH and activated LHR signaling results in increased ERK1/2 activation, lipid accumulation and testosterone production, leading to granulosa cell death and cyst formation.
Discussion

The PKB/Akt kinases are active in diverse physiological functions and have been shown to be important for the activity of various hormones. PCOS is associated with deregulation of LH signaling at various levels of the HPO axis and the resulting hyper-production of androgens (Goodarzi et al., 2011). As IR and compensatory hyperinsulemia are observed in 50-70% of PCOS patients (Diamanti-Kandarakis et al., 2008) and the beta isoform of protein kinase B (PKBβ/AKT2) is crucial for insulin signaling (Cho et al., 2001b; Garofalo et al., 2003; Dummler et al., 2006), this indicates deregulated activity of this isoform may be an important factor in this disease. Although PKB/Akt has been shown to be activated upon acute LH stimulation in thecal-interstitium (Lima et al., 2006), its subsequent contribution to androgenesis and PCOS pathology has remained unclear.

Here we report that aged PKBβ/AKT2 KO mice develop ovarian cysts associated with enhanced thecal steroidogenesis and hyperinsulemia. This effect was PKBβ/AKT2 isoform specific. However, in a young mouse model of PCOS, the ovaries of young animals lacking PKBβ/AKT2 and treated with LH displayed enhanced steroidogenesis and developed a cystic area threefold greater than WT controls, but with no indication of hyperinsulemia. Thus, the increased severity of cyst formation in young PKBβ/AKT2 ablated mice was not due to “co-gonadotrophin” stimulatory effects of hyperinsulemia (Poretzky et al., 1992) and loss of PKBβ/AKT2 appears to predispose mice to ovarian cysts independent of hyperactive insulin signaling.
Numerous studies have linked ovarian cyst development to increased LHR signaling and subsequent testosterone biosynthesis in the thecal-interstitium of the ovary (Chang, 2007). Postmenopausal women display follicular exhaustion that results in decreased conversion of testosterone to estrogen and an increase in LH due to loss of negative feedback to the pituitary (Choi et al., 2007). An involvement of LHR deregulation in cyst development is suggested by mouse models with disrupted LH expression. Mice overexpressing LH, like PKBβ/Akt2 knockout mice, display bilateral ovary involvement, thecal hyperplasia, increased testosterone levels and cyst development, and cyst development is also found in LHR KO mice (Danilovich and Ram Sairam, 2006; Huhtaniemi et al., 2006). However, circulating LH was detected in aged mice in this present study, with no significant difference between WT and PKBβ/Akt2 KO mice. Thus in this setting, only the specific loss of PKBβ/Akt2 led to severe cyst development with cyst development independent of deregulated LHR activation due to differences in LH levels.

Our results suggest that the single consistent consequence of the loss of specific PKBβ/Akt2 functions in aged ovaries that could contribute to cyst development is exacerbated androgenic signaling. At least in part, the effect of the ablation of PKBβ/Akt2 appears to be equivalent to the loss of functions controlling ERK activation and lipid accumulation, which leads to increased testosterone production. The identification in vivo of activated ERK, which was correlated with severe cyst formation in aged mice, and its high expression specifically in thecal cells adjacent to cysts in young mice in the PCOS model, suggests that inhibitors of ERK currently in clinical trials may have applications in treating ovarian cyst development in PCOS. However, it has been reported that ERK signaling is lost in thecal cells derived from ovaries of PCOS patients (Nelson-Degrave et al., 2005). This may reflect a difference
between the PCOS mouse model and the greater complexity of PCOS in human patients, or a difference between thecal cell signaling in the ovarian environment and that in isolated PCOS thecal cells \textit{in vitro}.

It remains to be seen whether as yet undefined PKBβ/Akt2 substrates in the ovary control hyper-androgenic production and loss of this signaling promotes PCOS, or alternatively whether its is inappropriate compensatory signaling by other PKB/Akt isoforms, in particular PKBα/Akt1, which is markedly expressed in the ovary and promotes pro-androgenic signaling. Opposing functions of PKBα/Akt1 and PKBβ/Akt2 have been reported in various tissues (Heron-Milhavet et al., 2006; Yun et al., 2008), as have isoform-specific interactions in the ovary (Nechamen et al., 2007). Solving this question will be crucial to the potential targeting of PKB/Akt signaling therapeutically in PCOS.

In conclusion, the results of this study highlight for the first time \textit{in vivo} a novel and specific effect of the loss of PKBβ/Akt2 on the development of ovarian cysts in the environment of pathogenic increased LHR signaling, and demonstrate \textit{in vivo} that activation of ERK in thecal cells is strongly associated with lipid accumulation and cyst development (as depicted in Figure 5). These results spotlight ERK inhibition and effectors downstream of PKBβ/Akt2 that display loss of function in an environment of increased LH in ovarian thecal cells as potential targets in the treatment and management of ovarian cysts and PCOS.

**Methods**
Reagents

Human LH (Lutophin) was obtained from Provet (Lyssach, Switzerland). The gonadotrophin-releasing hormone antagonist (GnRHAnt) was generously provided by Dr Jean Rivier (The Salk Institute, San Diego, Calif.).

Mice

The PKBα/Akt1 and PKBβ/Akt2 mutant mice, as described previously (Yang et al., 2003; Dummler et al., 2006), were housed in groups with 12-h dark-light cycles and with access to food and water ad libitum, in accordance with the Swiss Animal Welfare Laws. Matched WT and KO female mice aged 21-28 days were housed together to promote synchronous estrous cycling. PCOS induction experiments were commenced at ~28 days. All procedures were conducted with the appropriate approval of the Swiss authorities.

Tissue Preparation For Histology

For histological analysis, organs were dissected and either immediately gently snap-frozen in OCT compound in a 2-methylbutane bath in dry ice or fixed in 4% paraformaldehyde (PFA)-phosphate buffered saline (PBS). Tissues placed in 4% PFA-PBS were allowed to fix overnight (~18h) at 4°C. Tissues were then subjected to washes with PBS, 50% ethanol (EtOH)/PBS and 70% EtOH/PBS before being processed and embedded in paraffin using the Medite TPC15 Paraffin Processing Unit (Medite, Wintergarden, Fla.). Histological staining and immunohistochemistry (IHC) were performed on 12-μm frozen or 4-μm paraffin tissue sections, cut using a HM560H cryostat or M355S microtome (Thermo scientific, Fremont, Calif.).

Histological Staining
For haematoxylin and eosin (H&E) staining, sections were deparaffinized and stained according to the standard protocols using reagents purchased from Sigma (St. Louis, Mo.). Histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was carried out according to a modified protocol of Klinefelter et al. (Klinefelter et al., 1987). Briefly, 12-μm ovarian sections were cut on poly-L-lysine-coated glasses slides (Menzel-Gläser, Braunschweig, Germany) and covered with a staining solution prepared by mixing equal volumes of solution A: nitroblue tetrazolium (NBT) (#N6639, Sigma, St Louis, Mo.) and dehydroepiandosterone (DHEA) (#D1629, LKT Laboratories, St. Paul, Mn.) in PBS pH 7.4, with solution B: β-nicotinamide adenine dinucleotide (β-NAD) (#N7004, Sigma, St Louis, Mo.) in PBS pH 7.4. Final concentrations were 0.25 mM NBT, 1.5 mM β-NAD, 0.2 mM DHEA. Tissue slides were stained for 90 min at 37°C and fixed in 10% formalin in PBS with 5% sucrose, pH 7·4 at 4°C for 5 min. Slides were then rinsed in distilled water and counterstained for 5 min with Nuclear Fast Red (#H-3403, Vector Laboratories, Burlingame, Calif.). After rinsing again with distilled water, the sections were mounted and photographed under the microscope. Staining of lipids with Oil Red O was performed using the propylene glycol (PG) method: 12-μm fresh frozen sections were cut and air-dried at room temperature (RT) before being fixed at 4°C in 10% formalin for 5 min. Sections were then rinsed three times in ddH2O, allowed to air dry at RT, and then placed in 100% PG for 5 min before staining for 15 min with 0.5% (w/v) Oil Red O solution in PG pre-warmed to 60°C. Oil Red O solution was prepared by dissolving Oil Red O in PG at 90°C, filtering and allowing to stand at RT overnight. Staining was differentiated in 85% PG for 5 min and then rinsed twice with double distilled water (ddH2O). Slides were then counterstained with Gill’s Haematoxylin (#GHS216, Sigma, St Louis, Mo.) for 15 sec, rinsed three times in tap water, soaked in ddH2O for 5 min and mounted.
**Immunohistochemistry**

Sections of 4 μm were cut from paraformaldehyde-fixed, paraffin-embedded tissues and stained using the Ventana Discovery automated immunostainer (Ventana Medical Systems, Tucson, Az.). IHC was performed with or without cell conditioning using buffers CC1 or CC2, blocked with 5% normal donkey, goat or sheep serum for 1 hr. Primary antibodies diluted in Ventana antibody diluent were then applied and allowed to incubate for 1-12 hr at 25°C. Primary antibodies and dilutions used were vimentin (#V2009) 1:100 (Biomedia, Foster City, Calif.), Muellerin inhibiting substance (MIS, sc-6886) 1:1000 (Santa Cruz Biotechnology, Santa Cruz, Calif.), pCREB S133 (#9198) 1:100, pERK1/2 T202Y204 (#4370) 1:125 (both Cell Signaling Technologies, Danvers, Mass.). After washing, sections were incubated with biotinylated donkey anti-mouse (#715-067-003) or anti-rabbit (711-067-003) secondary antibodies (Jackson Immuno Research Inc, West Grove, Pa.) for 32 min at 37°C, before detection with HRP/DAB or UltraMap conjugates and counterstained with haematoxylin (all Ventana Medical Systems, Tucson, Az.). Photomicrographs were taken on a Nikon Eclipse E600 microscope (Nikon, Milville, N.Y.).

**Serum Hormone Measurement**

Blood samples were collected by sublingual vein puncture into Microvette CB300 tubes (Sarstedt, Nümbrecht, Germany) and serum separated by centrifugation at 5000g for 10 min. To account for the daily variation in hormones levels, a sample was taken in the morning and a second sample 6 h later. Aliquots (50 μl) of each sample were then pooled and concentrated to 50 μl by diethyl ether hormone extraction as described by (Wijayagunawardane et al., 2003). Concentrated hormone samples were then used in commercial enzyme-linked immunosorbent assay (EIA) kits (DRG...
Instruments GmbH, Marburg, Germany) to measure serum testosterone levels according to the manufacturer's instructions. For ultrasensitive insulin (Mercodia, Uppsala, Sweden) and LH (Endocrine Technologies Inc. Newark, Calif.) elisas, 25 µl of unconcentrated serum was used directly according to the manufacturer’s instructions.

**PCOS Induction and Cyst Measurement in Mice**

Mice were subjected to the standard protocol for LH-induced PCOS by injection of 0.05IU/gm hLH twice daily, with or without GnRHa Ant for 21 days as described previously. (Bogovich and Richards, 1982; Bogovich, 1987). At day 21, mice were sacrificed and samples collected for further analysis. Formation of haemorrhagic cysts, observed in mice treated with LH +/- GnRHa Ant, was quantified by sectioning through the ovaries and measuring every 100 µm the percentage of the total ovary area occupied by haemorrhagic cysts using ImageAccess Enterprise v10 software (Imagic Bildverarbeitung, Glattbrugg, Switzerland).

**TRANSLATIONAL IMPACT**

**Clinical Issue**

1. Ovarian cyst formation and PCOS are the most prevalent ovarian abnormalities affecting women, with significant social and economic impacts resulting from management and the often demanding invasive treatment.

2. PCOS is the most common cause of infertility and is associated with increased risks of other pathologies such as cardiovascular disease and physiological syndromes.

3. Increasing obesity/IR prevalence and later age of pregnancy in the female population is predictive of increased PCOS incidence and associated pathologies.

**Results**
1. Specific loss of PKBβ/Akt2 in mice predisposes animals to the formation of severe ovarian cysts.

2. Use of a rodent model of PCOS, with chronic LH administration to stimulate androgen production and ovarian cyst formation, resulted in more severe cyst formation in PKBβ/Akt2 mice than in WT.

3. Ovarian cyst formation in aged mice and PCOS-induced mice displayed increases in androgen-producing cells, lipid accumulation, ERK activation and increased circulating testosterone levels.

**Implications and Future Directions**

1. Indicates further studies into the effects of inhibitors of ERK signaling currently in clinical trials and/or identification of targets responsible for the control of excess androgen production downstream of PKBβ/Akt2. This may provide novel therapeutics to manage disorders involving ovarian cyst formation like PCOS.

2. Highlights ovarian cyst formation/PCOS as a potential side-effect of non-specific therapeutics currently in development that target PKB/Akt.

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**COMPETING INTERESTS**

The authors declare no competing interests.

**AUTHOR CONTRIBUTIONS**

All work was performed in the lab of B.A.H, who also provided theoretical input into the design and execution of this study. D.H. managed the mouse colonies, experimental licenses and proof-read the manuscript. D.H. and D.F.R performed aged necropsies and sampling. All other work and the assembly of the manuscript was performed by D.F.R.
References


Supplemental Figure 1: Aged PKBβ/Akt2 KO Ovaries Show An Absence Of Granulosa Cells Indicating Follicular Exhaustion. (A) Staining with anti-mullerian inhibiting substance to illustrate granulosa cells in aged PKBβ/Akt2 KO ovaries were completely negative, illustrating an absence of granulosa cells. Magnification 40x (i, ii) and 200x (iii, iv). (B) Immunohistochemistry for anti-mullerian inhibiting substance showing specificity and positive reactivity of the antibody specifically to granulosa cells. No background was observed in the conditions used when no antibody was applied (i-iv), nor was non-specific reactivity observed using testis negative control tissue. In contrast robust and specific staining was observed in the ovary solely in granulosa cells. Magnifications 40x (i, iii, v, vii) and 200x (ii, iv, vi, viii).
Supplemental Figure 2: Young PKBβ/Akt2 KO Mice Do Not Display Hyperinsulemia Compared To WT Animals. Analysis of serum from 4-8 week old mice show no increased insulin levels in PKBβ/Akt2 KO, indicating effects on cyst formation after LH administration are independent of hyperinsulemia developing in older PKBβ/Akt2 KO mice.
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